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Residual dipolar couplings

van Lune, Frouktje Sapke

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Chapter 1

Introduction

Membrane Transport Systems

Bacteria separate their interior from their surroundings by a cell envelope. In Gram-positive bacteria this envelope consists of a cytoplasmic membrane surrounded by a mechanically rigid and rather porous cell wall (murein or peptidoglycan layer). Gram-negative bacteria (which include *Escherichia coli*) have in addition to a cytoplasmic membrane and a thin cell wall one more membrane, the outer or periplasmic membrane (lipopolysaccharide layer), that is located outside the cytoplasmic membrane and the thin murein layer.

The murein layer, which confers bacteria mechanical support and prevents them from bursting from their high internal osmotic pressure, is an efficient barrier, that protects Gram-negative bacteria from a number of harmful compounds, such as some antibiotics, detergents and disinfectants. Both the outer and inner membranes are less porous and contain transport proteins that mediate the passage of a limited range of solutes.

The outer membrane contains three types of channels; porins, porin-like proteins and TonB-dependent receptors. Porins are large open water-filled channels, which non-specifically mediate the passive transport of small (< 600 Da), hydrophilic molecules and ions down their concentration gradient (Cowan *et al*, 1992; Schirmer *et al*, 1995; Weiss *et al*, 1991). Porin-like proteins contain more specific binding sites. The higher specificity results in an efficient facilitated diffusion of molecules that are present at low concentration in the environment. Large nutrients that exist in very low concentration outside the cell are actively transported across the outer membrane by a system composed of a high affinity outer membrane receptor and the TonB protein.

In contrast with the outer membrane, the cytoplasmic membrane is very selective. It contains transport proteins (carriers or permeases) that enable the cell to accumulate nutrients or excrete products against a concentration gradient. This type of transport is an energy requiring process. Based on the different energy coupling mechanisms the carriers use, they can be divided in the following four groups (Nikaido & Saier, 1992).

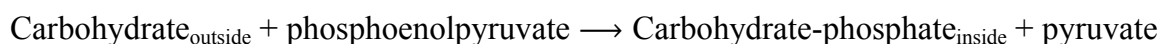
1. Facilitated diffusion: A specific protein facilitates the diffusion of a substrate across the membrane. This type of transport requires no external energy input other than the downhill substrate concentration gradient.
2. Primary transport systems: These transport systems use light or chemical energy to maintain and establish an electrochemical gradient across the membrane. Examples are the ABC transporters, the ATP-ases and the light driven bacteriorhodopsin proton pump.
3. Secondary transport systems: These transporters derive their energy from simultaneous downhill transport of other substrates across the membrane. The driving force is the electrochemical gradient established by the primary transport systems.

Examples are the lactose transporters and members of the Major Facilitator Superfamily, which use H^+ or Na^+ to accumulate or excrete solutes.

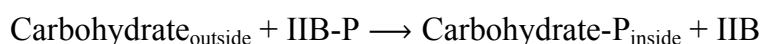
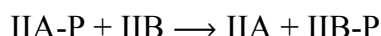
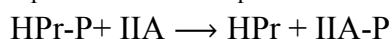
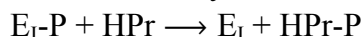
4. Group translocation systems: In these systems the translocation of the substrate across the membrane is coupled with an enzymatic reaction, resulting in a chemical modification of the substrate. An example of this class is the phosphoenolpyruvate-dependent phosphotransferase system (PTS). In this case, chemical modification of the substrate means phosphorylation of the carbohydrate during transport.

The phosphoenolpyruvate-dependent phosphotransferase system

The phosphoenolpyruvate-dependent phosphotransferase system, which was discovered by Kundig, Ghosh and Roseman (Kundig *et al*, 1964), is the main transport system for carbohydrates in Gram-positive and Gram-negative bacteria. The overall reaction carried out by the phosphotransferase system is:



This reaction is accomplished in five sequential, fully reversible phosphoryl group (P) transfer steps, which involve the two general cytoplasmic proteins, Enzyme I (E_I) and the histidine containing protein (HPr), and the carbohydrate specific Enzyme II complex (E_{II}) (figure 1).



The first phosphorylated intermediate is Enzyme I, a cytoplasmic component of 59 to 64 kDa. It is phosphorylated on a histidine by PEP, a step in which pyruvate is generated. Enzyme I then transfers the phosphoryl group to HPr, the smallest PTS protein (9-10 kDa) which, like Enzyme I, is phosphorylated on a histidine residue. Phosphorylated HPr in turn can phosphorylate all Enzyme II's. Contrary to the Enzyme I and HPr, the Enzyme II is specific for one or a few carbohydrates ($E_{II}^{\text{carbohydrate}}$). In a particular Enzyme II complex three or four functional units can be distinguished; two cytoplasmic domains of similar size (IIA and IIB) and one or two transmembrane domains (IIC and IID). These subunits occur either as a domain of a single polypeptide chain, or as separate polypeptides in almost any possible combination (Saier & Reizer, 1992; Siebold *et al*, 2001). The cytoplasmic domain IIA, a polypeptide usually of 100 to 160 amino acid residues, is phosphorylated from P-HPr at the active site histidine. Subsequently, IIA-P phosphorylates IIB at either a cysteine (in most PTS systems) or a histidine residue (mannose family). Finally, IIB activates IIC-dependent carbohydrate transport across the

cytoplasmic membrane with concurrent phosphorylation of the carbohydrate. The IIC domain is not phosphorylated. If present, the IID domain interacts with IIC. The phosphorylated carbohydrate is released in the cytoplasm where it can be used as a first intermediate in bacterial metabolism (figure 1) (Kotrba *et al*, 2001).

Enzyme II complexes have been divided into five classes based on their amino acid sequence and functional homologies: the glucose-sucrose family, the mannose family, the mannitol-fructose family, the glucitol family and the lactose-chitobiose family (Cornilescu *et al*, 2002). Relations between different Enzyme II complexes are reflected in their sequence and in their domain structure. Sequence identities within one family are usually greater than 25% and a component of an Enzyme II complex can often be complemented by a homologous component from the same family without severe loss of activity (van Montfort *et al*, 1997). Between components of different families, however, the sequence similarities are mostly limited to the phosphorylation sites.

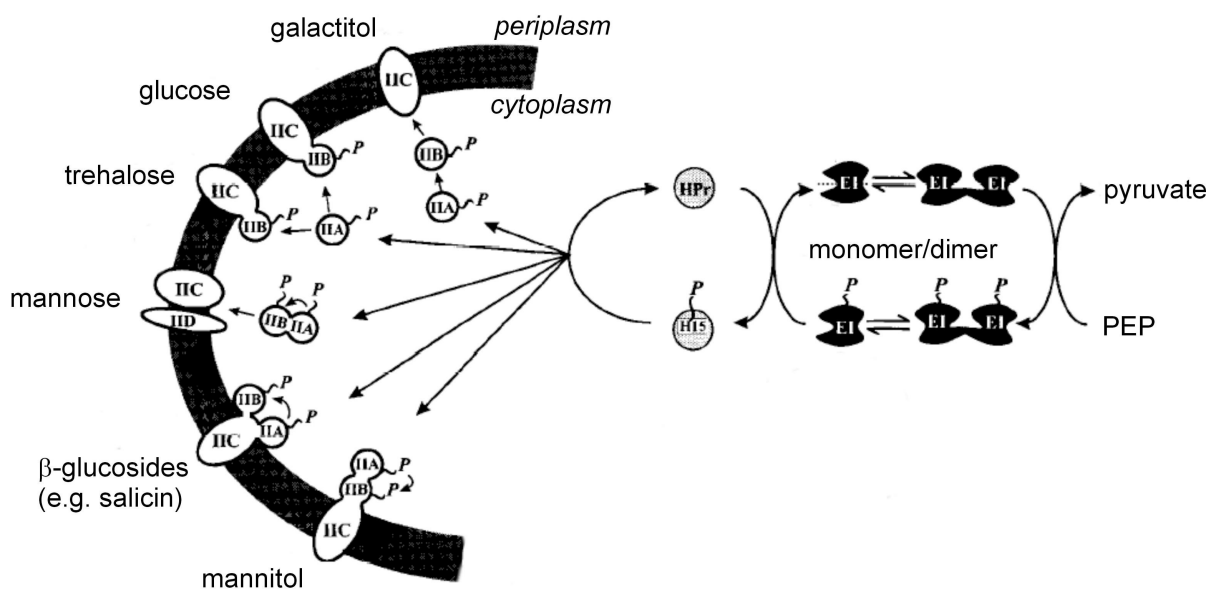


Figure 1: A schematic representation of the PTS (Kotrba *et al*, 2001). The figure shows the common elements Enzyme I and HPr, and the Enzyme II of several carbohydrate specific EIIs from E.coli. The transported carbohydrate is indicated on the outside the cell. Arrows indicate the route of the phosphoryl-group transfer.

In addition to its transport function, the PTS has also some regulatory functions (Saier & Reizer, 1994). The PTS as a whole has at least three functions (Mao *et al*, 1995):

1. Uptake of carbohydrates coupled to their phosphorylation
2. Regulation of metabolic pathways like catabolic repression and inducer exclusion.
3. Chemotaxis.

The main mechanisms by which PTS regulation operates is phosphorylation and dephosphorylation of the PTS proteins Enzyme I, HPr and IIA^{glc}. Depending on their

phosphorylation state they bind to other enzymes and thus regulate the processes in which the enzymes are involved.

Catabolite repression is a process by which the expression of many catabolic genes is repressed when carbon sources that are more favourable are available. In Gram-negative bacteria IIA^{glc} plays an important role in the catabolite repression. Interaction of phosphorylated IIA^{glc} with adenylate-cyclase results in an inactivation of the enzyme and formation of cyclic-AMP (cAMP). The level of cAMP in the cell is an important signal in the regulation of gene expression (Eppler *et al*, 2002; Hogema *et al*, 1998).

In Gram-negative bacteria, IIA^{glc} is also involved in a cAMP independent form of catabolite repression called inducer exclusion. Unphosphorylated IIA^{glc} allosterically inactivates various catabolite transporters and enzymes by binding to these proteins. Once inactivated, these systems are no longer able to influence the import of inducer molecules. Consequently, expression of these uptake systems is not stimulated even when the substrate is available in the medium (Hogema *et al*, 1998).

HPr, rather than IIA^{glc} , is the key regulatory protein of catabolite repression and inducer exclusion in Gram-positive organisms. In addition to histidine 15, which is phosphorylated in a PEP-dependent reaction catalysed by Enzyme I, HPr of Gram-positive bacteria can be phosphorylated on a second phosphorylation site, serine 46, which is a regulatory site. Phosphorylation is accomplished by an ATP dependent kinase. The activity of this kinase is stimulated by metabolic intermediates, for example fructose-1,6-biphosphate (an intermediate of the glycolysis) and repressed by inorganic phosphate. When rapidly catabolizable carbon sources are available, the level of fructose-1,6-biphosphate will rise and the kinase in turn will phosphorylate HPr on serine 46. Under starvation conditions the process will be the reversed and consequently P-Ser-HPr will be dephosphorylated (Saier & Reizer, 1994). Phosphorylation of HPr on serine 46 has two major effects (Deutscher *et al*, 1995; Hueck *et al*, 1994)

1. It competes with phosphorylation on histidine 15 by Enzyme I, resulting in the decrease of PTS-dependent uptake of carbohydrates.
2. A complex will be formed with the catabolite control protein A (CcpA). When this complex binds to catabolite response elements (CRE), located in or near promoters of many catabolic operons, expression of the subsequent genes is sterically blocked.

P-Ser-HPr is, like IIA^{glc} in Gram-negative bacteria, involved in the inducer exclusion in Gram-positive bacteria by allosterically inactivating non-PTS transporters, thereby preventing the uptake of inducer molecules (Saier *et al*, 1996). Another process in which P-Ser-HPr is implicated is inducer expulsion, in which pre-accumulated carbohydrate-phosphates are dephosphorylated by a phosphatase and subsequently transported out of the cell. Inducer exclusion as well as inducer expulsion regulate the enzyme synthesis in the catabolism of less-advantageous sugars by modulation of the intracellular inducer concentration. However, the inducer exclusion mechanisms is indirect and relatively slow in contrast to the inducer expulsion mechanism which

modulates the intracellular inducer concentration directly and instantaneously (Reizer & Panos, 1980; Ye *et al*, 1996).

The last function of the PTS is chemotaxis, the directed movement of bacteria toward some specific substances and away from others. In this process the direction of the flagellar rotation is directed by phosphorylation or dephosphorylation of the *che* genes. The uptake of carbohydrates leads to dephosphorylation of Enzyme I, which in turn inhibits the autophosphorylation of CheA, the kinase of CheY. Consequently, the levels of phosphorylated CheY decrease and a smooth up-gradient swimming response will be the effect (Garriety *et al*, 1998; Lux *et al*, 1995).

Protein structure determination using NMR

To understand the mechanism of carbohydrate transport and the regulatory processes in which the PTS is involved it requires knowledge of the three-dimensional structures of the components of the PTS. With the availability of high-resolution three dimensional structures of an increasing number of PTS proteins, the possibility of rational drug design (Martin, 1991), using these proteins as targets, is worth considering. The value of this and other approaches that take advantage of the unique features of the bacterial PTS, lies in the fact that the PTS is prokaryotic specific. Because the PTS has not been found in eukaryotes, and because it phosphorylates numerous sugars that are not phosphorylated by eukaryotic sugar kinases, it may also serve as a vehicle for the entry into prokaryotes of novel classes of antibiotics (Parr & Saier, 1992).

In recent years, detailed structural studies have been carried out on some PTS components, but for others only scarce or no structural information is available. Two widely used mechanisms to determine a protein structure are X-ray crystallography and Nuclear Magnetic Resonance (NMR). This thesis focuses on the latter and in particular on the use of dipolar couplings to refine and determine protein structures.

Traditionally, the NMR approach to structure determination of biomolecules has been based on the interpretation of a large number of semi quantitative local restraints. The most important of these is the ^1H - ^1H NOE (Nuclear Overhauser Effect), which provides distance information for pairs of protons separated by less than ~ 5 Å (Bax, 2003). The NOE data can be supplemented by a number of other restraints that are dependent on the close spatial proximity of atoms, e.g. three-bond scalar couplings and chemical shifts (Clore & Schwieters, 2002).

While chemical shifts and J-coupling constants give local structure information, NOE data can relate atoms that are far apart in the series of chemical bonds connecting the biomolecule, but are close in space. Usually, structure determination requires a large amount of NOE data. The typical qualitative interpretation of NOE data can lead to structures with acceptable precision only because the inherent redundancy in the data.

In spite of this, there are some cases where all the traditional NMR parameters fail to determine the three dimensional structure. One example is when a protein is not globular and has several domains that are structurally independent. In this situation it is very difficult to determine the relative orientation of the domains, due to the sparse

number of NOE crosspeaks connecting them (Clore & Schwieters, 2002; de Alba & Tjandra, 2002). Another example is when the NMR signals are not assigned to individual protons of specific residues in a known protein sequence, because the structure determination using NOE data relies on having first assigned a substantial fraction of the ^1H spectrum.

The assignment of resonances and measurement of adequate numbers of NOEs have always been obstacles that made structure determination time-consuming and limited to relatively small proteins (< 10,000 Mw for early homonuclear studies). The limitations have been pushed back over the years with additional structural information from scalar coupling constants and chemical shifts. Additionally, assignment strategies based on the use of through bond connectivities between ^{13}C and ^{15}N sites in isotopically enriched proteins have also made it possible to assign resonances in increasingly larger proteins. However, full structure determinations have still remained confined to reasonably compact systems of molecular weights less than 40,000 Mw (Clore & Gronenborn, 1997; Wagner, 1997).

Recently, a different source of structural information has been used for structure determination: the magnetic dipole-dipole coupling between spin $\frac{1}{2}$ nuclei (e.g. ^1H , ^{13}C , ^{15}N). These dipolar couplings contain information on the orientation of inter-nuclear vectors relative to the magnetic field and have proven invaluable for improving the accuracy of macromolecular NMR structure determination, for independently validating this accuracy, for refining crude homology of proteins and for defining intermolecular interactions. In addition, dipolar couplings can be used to search for homologous structures or substructures in a structure database, and potentially could replace the time consuming regular NOE data collection and analysis process (Gaemers & Bax, 2001).

Residual dipolar couplings

The use of residual dipolar couplings (RDC) to enhance the information available from high-resolution NMR spectra has a long history. Its roots can be traced to the substantial amount of NMR done in magnetic-field-aligned liquid crystals in the early 1960's by Saupe and Englert (Saupe & Englert, 1963). They demonstrated that an organic molecule dissolved in a nematic liquid crystalline phase exhibits quite strong alignment when placed in an NMR magnet. Thoughts about application to biomolecules in solution arose more than 15 years ago with the observation that isolated molecules with sufficient anisotropic susceptibilities would adopt slightly non-isotropic orientational distributions when placed in a high magnetic field. The major breakthrough with respect to any potentially routine use of dipolar couplings for biomolecular structure determinations was the demonstration that adjustable degrees of alignment could be achieved by placing the molecule under investigation into a dilute, aqueous liquid crystalline phase of dihexanoyl phosphatidylcholine (DHPC) and dimyristoyl phosphatidylcholine (DMPC) (Tjandra & Bax, 1997).

The application described above relies on the fact that anisotropic contributions to nuclear interactions, e.g. dipolar interaction between pairs of magnetically active spin $\frac{1}{2}$

nuclei, do not average to zero when the molecules of interest have a preferred orientation. The net alignment of the molecules of interest, which can be introduced by a liquid crystalline medium, is on the order of 10^{-3} and is fundamental to the success of RDC-based studies.

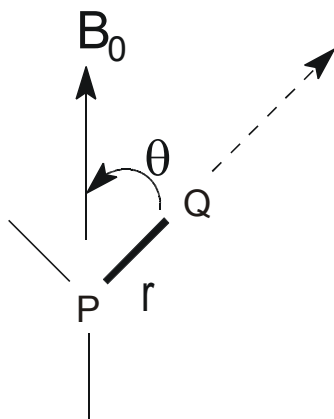


Figure 2: Dipolar coupled spin pair. The bond length r is assumed to be fixed and the primary variable is the angle θ between the magnetic field B_0 and the internuclear vector.

Dipolar couplings are potentially quite large interactions, caused by the magnetic field produced by one nucleus (e.g. nucleus Q) affecting the energy of another nucleus (e.g. nucleus P) (figure 2). The components orthogonal to the magnetic field B_0 have a negligible effect on the total magnitude of the vector sum of the external and the dipolar field. Thus only the z component of the dipolar field of nucleus Q will change the resonance frequency of nucleus P by an amount that depends on the internuclear distance and on the orientation of the internuclear vector relative to B_0 . For a fixed orientation of the vector, nuclear spin P can decrease or increase the total magnetic field at nucleus Q, depending on whether P is parallel or antiparallel to B_0 . In an ensemble of molecules half of the P nuclei will be parallel to B_0 and the other half antiparallel, and Q will show two resonances separated in frequency by

$$(1) D^{PQ} = D_{\max}^{PQ} \langle (3\cos^2\theta - 1)/2 \rangle$$

where θ is the angle between the internuclear vector and B_0 , the brackets $\langle \rangle$ denote time or ensemble averaging, and

$$(2) D_{\max}^{PQ} = \frac{\mu_0}{4\pi} \gamma_1 \gamma_2 \frac{\hbar}{2\pi} \left\langle \frac{1}{r_{PQ}^3} \right\rangle$$

is the doublet splitting that applies for the case where $\theta = 0$. The meaning of the other symbols is: μ_0 = magnetic permittivity of vacuum; $\hbar = h/(2\pi)$, in which h = Planck's constant; γ_P , gyromagnetic ratio of nucleus P; γ_Q , gyromagnetic ratio of nucleus Q; r_{PQ} =

the distance between nucleus P and Q. Equation 1 shows that the dipolar splitting, D^{PQ} , provides direct information on the angle θ .

Knowing θ for a bonded pair of nuclei, e.g. ^1H - ^{15}N , can be very useful in defining a molecular structure. The brackets around the θ dependent term, however, denote a time average. Normally in solution we assume that the time average results from a molecular tumbling that uniformly samples directions in space (isotropic sampling). Consequently, for any pair of nuclei, the $3\cos^2\theta-1$ term averages to zero and no residual dipolar coupling can be measured (Prestegard, 1998). For this reason NMR spectroscopists were relegated to measure dipole-dipole interaction indirectly through spin relaxation based phenomena such as the NOE until 1997, when Tjandra and Bax demonstrated the use of a liquid crystalline medium to introduce a tunable degree of alignment.

It is important to introduce a controllable, but small amount of alignment. Under these conditions, the rotational diffusion of the protein is more-or-less unhindered, thus maintaining the narrow linewidth typical of liquid NMR. The partial alignment (approximately one part in 1000) results also in a net alignment which allows the measurement of residual dipolar couplings only for those nuclei that are very strongly coupled. So, the small tunable degree of alignment provides the benefits of both solid-state (structural information) and liquid-state (narrow lines) spectroscopy.

Residual dipolar couplings also can be measured in the absence of any liquid crystalline medium when the molecule of interest has a sufficiently large intrinsic anisotropy of its magnetic susceptibility to achieve a preferred orientation with respect to the external magnetic field B_0 (Tolman *et al*, 1995). This has been shown for both paramagnetic and diamagnetic biomolecules (Contreras *et al*, 1999). Even for paramagnetic proteins, the natural alignment with a high magnetic field is usually one order of magnitude smaller than that achieved by the use of an orienting medium. The same is true for nucleic acids, in which the stacking of their bases dominates the susceptibility anisotropy (Kung *et al*, 1995).

Measurement of residual dipolar couplings

One important property of residual dipolar couplings between two nuclei P and Q that have a scalar coupling is that the dipolar coupling (D^{PQ}) is added to the isotropic coupling constant (J^{PQ}). Therefore, when measuring the effective dipolar coupling under anisotropic conditions (E^{PQ}), the spin-spin coupling needs to be subtracted from the measured coupling to obtain the desired dipolar coupling.

$$(3) E^{PQ} = J^{PQ} + D^{PQ}$$

Often the one-bond dipolar couplings (e.g. NH , $\text{CaH}\alpha$, CaC' , $\text{C}'\text{N}$) are measured, but also two bond and three bond dipolar couplings can be measured (figure 3). One-bond dipolar couplings are easier to interpret because the inter-atomic distance is known and the magnitude of the dipolar interaction is relatively large (de Alba & Tjandra, 2002).

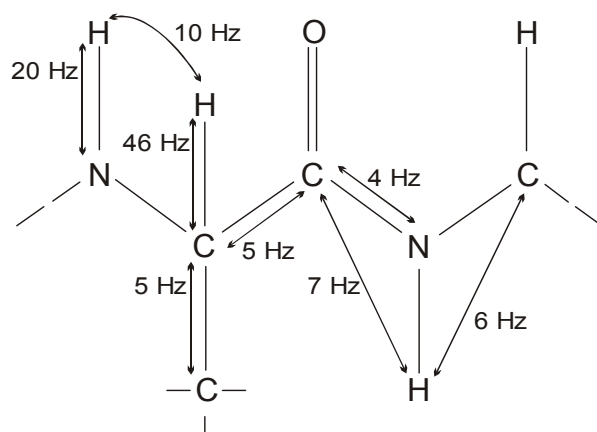


Figure 3: The predicted maximum absolute residual dipolar contributions for different nuclei in the protein main-chain at 0.1% levels of alignment.

The NMR methods used for measuring residual dipolar couplings can be divided into two general categories: frequency resolved methods (J-resolved) and intensity based experiments (J-modulated). In frequency resolved methods the separation of the peaks is measured in a frequency domain. This can be achieved, e.g., by omission of the normal 180° ^1H pulse in the middle of the heteronuclear evolution period of a heteronuclear single quantum coherence (HSQC) experiment, which provides spectra with the scalar and residual dipolar coupling splittings in the indirect dimension. This method is illustrated in figure 4a, which shows a selected region from a ^1H -coupled ^1H - ^{15}N HSQC spectrum. In intensity based experiments the coupling is extracted from the resonance intensity rather than from the experimental splitting (Figures 4b and 4c). The principle underlying the J modulated experiments is to pass the observed signal through a period in which the intensity is modulated by a known function of the spin-spin coupling (Brunner, 2001; Prestegard *et al*, 2000). An intensity-based experiment is particularly useful in case of overlap problems in the described coupled spectra because of the increased number of signals due to the doublet splitting. In addition, J-modulated spectroscopy is a good alternative when the coupling of interest is small compared with the line width, which is often the case with ^3J -couplings. Besides the advantages, the intensity-based experiments have one important disadvantage, the total acquisition time. For example, the J-modulated HSQC and the ^1H -coupled HSQC shown in figure 4 took 18 hours and 1.5 hour to record, respectively.

Outline of this thesis

To understand the complete PTS system, including the mechanism of transport and the mechanism of energy coupling to the PTS proteins, the three dimensional structure of the proteins involved has to be known. The aim of the thesis is to investigate how residual dipolar couplings can be used to resolve or refine the three-dimensional structure of one of the proteins of the PTS system.

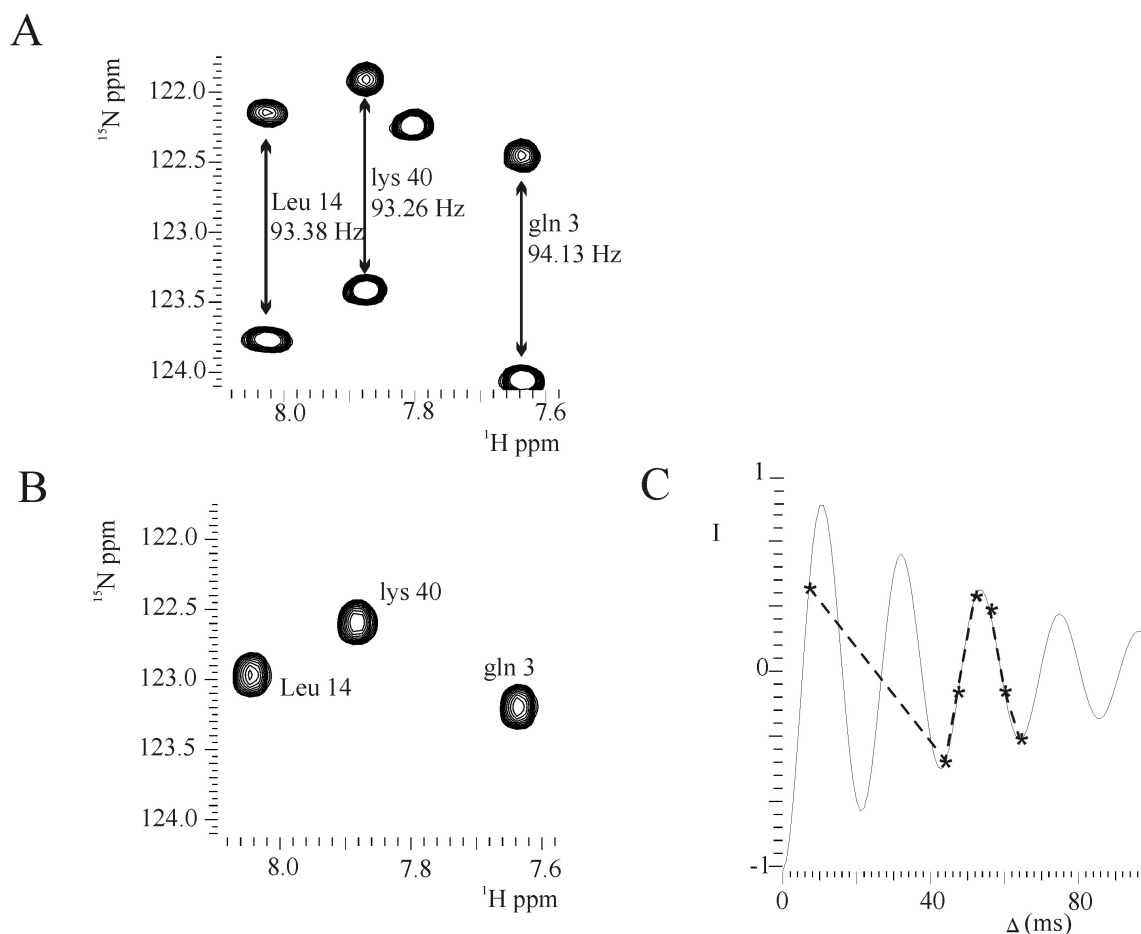


Figure 4: Small sections of the ^1H -coupled HSQC (a) and one of a series of the J-modulated HSQC (b) spectra of 2 mM HPr-WT recorded at 600 MHz, 293 K. Figure 4c shows the modulation of resonance intensity, I , for glutamine 3 as a function of the delay Δ . The best fit is plotted in black ($J=94.06$ Hz). The dotted line connects the measured intensities.

For measuring residual dipolar couplings, one needs an orienting medium. Chapter two describes the different media used for aligning biomolecules in a magnetic field. The properties, advantages and disadvantages of the different orienting media will be discussed to make it easier to find the most compatible medium.

Once a suitable medium has been found, one can measure residual dipolar couplings. Most sequences for the measurement of $^{13}\text{C}\alpha$ - $^1\text{H}\alpha$ and $^{13}\text{C}'$ - $^{13}\text{C}\alpha$ couplings are based on the HNCO and (HA)CA(CO)NH. However, in some cases (e.g. when the N_{i+1} resonance can not be measured) one needs a different type of pulse sequence. Chapter three describes a COCAH-based pulse sequence for measuring simultaneously $^{13}\text{C}\alpha$ - $^1\text{H}\alpha$ and $^{13}\text{C}'$ - $^{13}\text{C}\alpha$ couplings in ^{15}N - ^{13}C labeled proteins.

After the measurement of residual dipolar couplings in a protein of known three-dimensional structure, an order parameter tensor can be calculated. The order-parameter tensor gives information about the orientation of the biomolecule relative to the magnetic field. In chapter four, an order-parameter tensor description of HPr is given in an orienting medium of phospholipids.

In chapter five, the assignment of the backbone resonances of the protein IIB^{mtl} is presented. Of this protein, no three-dimensional structure is available at the moment.

Some suggestions are given for resolving the three-dimensional structure using residual dipolar couplings in chapter six.

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